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Phenalen-1-one mediated antimicrobial photodynamic therapy and chlorhexidine applied to a novel caries biofilm model

Fabian Cieplik[1,*], Fabian Wimmer[1], Denise Muehler[1], Thomas Thurnheer[2], Georgios N. Belibasakis[3], Karl-Anton Hiller[1], Tim Maisch[4] and Wolfgang Buchalla[1]

[1] Department of Conservative Dentistry and Periodontology, University Medical Center Regensburg, Regensburg, Germany

[2] Clinic of Preventive Dentistry, Periodontology and Cariology, Division of Oral Microbiology and Immunology, Center for Dental Medicine, University of Zurich, Zurich, Switzerland

[3] Division of Oral Diseases, Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden

[4] Department of Dermatology, University Medical Center Regensburg, Regensburg, Germany

Short title: aPDT and CHX for inactivation of biofilms formed by caries-associated bacteria

Key words: photodynamic, phenalen-1-one, biofilm, polymicrobial, caries, *Streptococcus mutans*, chlorhexidine

*** Corresponding author:**
Dr. Fabian Cieplik
Department of Conservative Dentistry and Periodontology
University Medical Center Regensburg
Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany
email: fabian.cieplik@ukr.de

Abstract

Antimicrobial photodynamic therapy (aPDT) may be useful as a supportive antimicrobial measure for caries-active subjects. In this study, the antimicrobial efficacy of aPDT with a phenalen-1-one photosensitizer is evaluated in a novel *in vitro* biofilm model comprising *Actinomyces naeslundii*, *Actinomyces odontolyticus* and *Streptococcus mutans* and compared to chlorhexidine. The proposed biofilm model allows high-throughput screening for antimicrobial efficacy while exhibiting a differentiated response towards different antimicrobial approaches. While chlorhexidine 0.2% showed reduction of ≈4 $\log_{10}$ for all species, aPDT led to more pronounced reduction of *S. mutans* (2.8 $\log_{10}$) than *Actinomyces* spp. (1.2 or 1.3 $\log_{10}$). A similar effect was also observed in monospecies biofilms. Therefore, aPDT may be more effective against *S. mutans* than *Actinomyces* spp. when in biofilms, and this antimicrobial approach merits further investigations.

Introduction

According to the Global Burden of Disease 2015 study, dental caries was still found to be the most prevalent disease worldwide, affecting about 2.3 billion adults and 560 million children [GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016] and, accordingly, may be considered as a major economic burden for public health care [Sheiham, 2001]. With regard to distribution of dental caries, it is well known that there are several disparities by social standing mostly because of differences in diet, fluoride use, and social empowerment, resulting in a sub-population with strong caries incidence [Edelstein, 2006]. Therefore, the development of prevention concepts for these high-risk patients remains a major goal.

In this regard, supportive antimicrobial measures may be useful in daily oral hygiene practice additionally to mechanical removal of biofilms for caries-active patient subgroups [Cate, 2009]. The widely used antiseptic of choice is chlorhexidine (CHX), either applied as oral rinse or as gel or varnish [Walsh et al., 2015]. However, its actual impact on caries prevention is debated controversially and its use exhibits some undesirable side-effects like yellow-brown staining of teeth and tongue and altering the sense of taste [Autio-Gold, 2008]. Furthermore, recently its use has been recommended to be limited to “those applications with a clear patient benefit” (mostly in intensive care) for reducing the risk of inducing acquired resistances to CHX or even cross-resistances towards antibiotics in pathogens [Kampf, 2016]. Accordingly, Kitagawa *et al*. reported that repeated exposure of *Enterococcus faecalis* to CHX resulted in drug resistance as demonstrated by increased minimum inhibitory concentrations [Kitagawa et al., 2016].

In this instance, the antimicrobial photodynamic therapy (aPDT) may be a promising alternative [Gursoy et al., 2013; Wainwright et al., 2017]. aPDT is a three-component system, consisting of a *per se* non-toxic dye (photosensitizer, PS), light of an appropriate wavelength and molecular oxygen. When the PS-molecule is activated by light, it transmits to an excited state, wherefrom there are two mechanisms to regain its ground state: In type I mechanism, charge is transferred to a substrate or molecular oxygen resulting in emergence of oxygen radicals like superoxide ions and hydroxyl radicals or hydrogen peroxide. In type II mechanism, energy is transferred to molecular oxygen, resulting in generation of singlet oxygen ($^1O_2$), which is regarded as the most effective reactive oxygen species [Wainwright, 1998; Cieplik et

al., 2014; Wainwright et al., 2017]. Hereby, the singlet oxygen quantum yield Φ_Δ describes the proportion of type II mechanism [Maisch et al., 2007]. Due to its unselective mechanism of action, the potential of resistance induction towards aPDT is very unlikely [Tavares et al., 2010; Giuliani et al., 2010], especially for type II mechanism [Cieplik et al., 2013]. Accordingly, PS based on a phenalen-1-one structure seem to be particularly advantageous due to their high singlet oxygen quantum yield $\Phi_\Delta \geq 0.95$ [Späth et al., 2014; Cieplik et al., 2013; Tabenski et al., 2016]. Furthermore, for oral application, esthetic compromises for patients will not be an issue due to the tooth-like color of phenalen-1-one derivatives [Späth et al., 2014]. However, before any clinical application it is mandatory to evaluate novel antimicrobial approaches for their efficacy against biofilms as it is well known that bacteria embedded in biofilms exhibit strongly enhanced tolerance towards antimicrobials challenges as compared to their planktonic counterparts [Marsh, 2004]. Accordingly, it is of vital importance to develop *in vitro* biofilm models that are easy to operate and allow high-throughput screening of given antimicrobial compounds or approaches.

The aim of the present study was to evaluate the antimicrobial efficacy of phenalen-1-one mediated aPDT on a novel polymicrobial biofilm model cultured from caries-associated bacteria *in vitro,* in comparison to the gold-standard antiseptic chlorhexidine.

Materials and Methods

Chemicals and light source

The PS used in this study was 2-((4-pyridinyl)methyl)-1H-phenalen-1-one chloride and had been synthetized at the Department of Organic Chemistry (University of Regensburg, Germany), as described earlier in detail (purity ≥ 99%) [Cieplik et al., 2013; Späth et al., 2014]. PS-suspensions were freshly prepared for the experiments and stored in the dark at 4°C for no longer than 2 weeks. For irradiation of the PS, a gas-discharge lamp (Waldmann PIB 3000; Waldmann Medizintechnik, Villingen-Schwenningen, Germany) was employed (λ_{em} 380–600 nm). Irradiance was adjusted to 50 mW/cm^2 at sample-level, resulting in an energy dose of 30 J/cm^2 for an irradiation period of 10 min. Chlorhexidine-digluconate dissolved in *aqua dest*. was

prepared by the pharmacy department of the University Medical Center Regensburg yielding concentrations of 0.2% and 2%.

Bacterial culture and biofilm formation

Three reference strains, *Actinomyces naeslundii* (DSM-43013), *Actinomyces odontolyticus* (DSM-19120) and *Streptococcus mutans* (DSM-20523) were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) to be used in this study. Bacteria were grown and maintained on Columbia Agar plates (provided by the Institute for Microbiology and Hygiene, University Medical Center Regensburg, Germany) in a microincubator (MI23NK, SCHOLZEN Microbiology Systems, Necker, Switzerland) under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). Modified Fluid Universal Medium (mFUM) supplemented with 67 mmol/l Sørensen's buffer (pH 7.2) and containing carbohydrate (0.15% (w/v) glucose and 0.15% (w/v) sucrose) was employed as a basal liquid medium [Guggenheim et al., 2001; Gmür, and Guggenheim, 1983]. For preparation of planktonic cultures, colonies were picked and suspended in 5 ml of mFUM with 0.5 ml fetal bovine serum (FBS; Gibco® life technologies, Carlsbad, CA, USA) for 24 h under anaerobic conditions to obtain bacteria in the stationary growth phase. Afterwards, suspensions were harvested by centrifugation (ROTINA 420 R, Hettich Lab Technology, Tuttlingen, Germany) and resuspended in mFUM yielding an optical density (OD) of 1.0, as measured by means of a spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Amersham, UK). Bacterial suspensions were diluted 1:9 in the biofilm culture medium (BCM) consisting of 50% mFUM, 10% FBS and 40% whole unstimulated human saliva (saliva) that had been pooled from two volunteers (authors FW, DM) and sterile-filtered (pore size: 0.2 µm; Acrodisc® Syringe Filters, Pall, Newquaw, UK).

Polyspecies biofilms were formed in 96-well polystyrene culture plates (Corning® Costar®, Corning, NY, USA). For simulation of pellicle coating, wells were incubated with saliva for 2 h at room temperature. After that, saliva was discarded, and wells were filled with 200 µl of BCM containing *A. naeslundii* and *A. odontolyticus* and incubated under anaerobic conditions. After 16 h, medium was carefully removed and 200 µl fresh BCM containing *S. mutans* was added. After 48 h, a further medium change was performed and biofilms were incubated for another 24 h.

Formation of monospecies biofilms was performed accordingly: after a 2 h incubation with saliva, wells were filled with 200 µl of BCM containing *A. naeslundii*, *A. odontolyticus* or *S. mutans*, respectively. Medium changes were after 24 h and 48 h. In all cases, the total culture period was 72 h.

Antimicrobial assay

After 72 h, medium was carefully discarded from the wells and the biofilms were either incubated with phosphate buffered saline (PBS; Biochrom, Berlin, Germany; groups PS-L-, PS-L+) or PS (groups PS+L-, PS+L+), respectively, in the dark for 10 min and then illuminated for another 10 min or incubated with CHX 0.2% or CHX 2% for a total of 20 min (50 µl each). Immediately afterwards, PBS, PS or CHX was carefully removed and each biofilm was brought to suspension with 200 µl of PBS and transferred to an Eppendorf tube. These were placed in an ultrasonic water-bath chamber (Sonorex Super RK 102 H, Bandelin, Berlin, Germany) obtaining a frequency of 35 kHz for 10 min and vortexed (REAX top, Heidolph Instruments, Schwabach, Germany) for 5 s to separate aggregated bacteria. Tenfold serial dilutions (10^{-2} to 10^{-7}) were prepared in PBS and aliquots (180 µl) were plated on Columbia blood agar and incubated anaerobically for 72 h. Afterwards, colony forming units (CFU) were evaluated. For polyspecies biofilms, bacteria on agar plates were differentiated by their respective colony morphology.

Data analysis

All results are shown as medians and neighboring quartiles (25/75% percentiles) and were calculated using SPSS for Windows, v. 23 (SPSS Inc., Chicago, IL, USA) from the values of at least six independent experiments, each performed in duplicate. Horizontal dotted and dashed lines represent reductions of 3 and 5 $\log_{10}$ steps of CFU, respectively, compared to the matching untreated control group PS-L-. Medians on or below these lines demonstrate antimicrobial efficacy of at least 99.9% (3 $\log_{10}$) or 99.999% (5 $\log_{10}$), respectively, which is declared as biologically relevant antimicrobial activity or disinfectant effect according to the guidelines of infection control [Boyce, and Pittet, 2002].

Results

Antimicrobial efficacy against polyspecies biofilms

Untreated polyspecies biofilms (PS-L-) showed an overgrowth of *S. mutans* ($4.7x10^7$) of ≈1 log_{10}-step as compared to *A. naeslundii* ($3.8x10^6$) and *A. odontolyticus* ($4.3x10^6$). The left panel of Figure 1 shows absolute CFU values of untreated controls while the right panel depicts relative CFU data with untreated controls (groups PS-L-) set to 100%.

aPDT reduced CFU of *S. mutans* (2.8 log_{10}) more than CFU of *A. naeslundii* and *A. odontolyticus* (1.2 or 1.3 log_{10}, respectively), while treatment with PS (PS+L-) or light alone (PS-L+) had no effect. CHX 0.2% reduced CFU of all species by ≈4 log_{10}-steps (*S. mutans*: 4.0 log_{10}; *A. naeslundii*: 4.5 log_{10}; *A. odontolyticus*: 3.8 log_{10}) while CHX 2% led to eradication by >6 log_{10} below the detection limit.

Antimicrobial efficacy against monospecies biofilms

Untreated monospecies biofilms (groups PS-L-) showed growth of $2x10^8$, $4x10^7$ and $1x10^6$ CFU for *S. mutans*, *A. naeslundii* and *A. odontolyticus*, respectively. Figure 2 shows relative CFU data with untreated controls (groups PS-L-) set to 100%.

CFU-reductions for aPDT (PS+L+) were 3.0 log_{10} for *S. mutans*, while *A. naeslundii* and *A. odontolyticus* were reduced by 2.5 log_{10} or 1.1 log_{10}, only. In contrast, CHX 0.2% led to reduction rates of 4.3 log_{10} for *S. mutans* and *A. odontolyticus*, while CFU of *A. naeslundii* were reduced by 6.0 log_{10}. Treatment with PS (PS+L-) or light only (PS-L+) had no effect in all cases.

Discussion

The aim of the present study was to investigate the antimicrobial efficacy of phenalen-1-one mediated aPDT as compared to the gold-standard antiseptic chlorhexidine for inactivation of biofilms from cariogenic bacteria *in vitro*. For this purpose, a novel biofilm model for high-throughput screening of antimicrobial approaches was established comprising *Actinomyces naeslundii*, *Actinomyces odontolyticus* and *Streptococcus mutans*. *A. naeslundii* is considered to be an essential early colonizer in dental plaque [Dige et al., 2009], while *A. odontolyticus* is suggested to be a key organism mediating adhesion and co-aggregation in

supragingival plaque [Tang et al., 2003] and is associated with high caries incidence in children [ElSalhy et al., 2016]. *S. mutans* is well-known to play a major role in pathogenesis of dental caries mostly due to its ability to produce an insoluble polymeric matrix and its acidogenic and aciduric properties [Klein et al., 2015].

The culture conditions for this biofilm model have been modified from the Zurich biofilm model of supragingival plaque originally described by Guggenheim and co-workers [Guggenheim et al., 2001]: Instead of pasteurization of human saliva, filter-sterilization was employed because the latter is deemed to be preferable in terms of protein integrity [Ruhl et al., 2011]. Furthermore, concentrations of carbohydrates in mFUM were changed from 0.15% glucose towards 0.15% sucrose and 0.15% glucose each, since *S. mutans* requires sucrose for glucosyltransferase-mediated synthesis of exopolysaccharides [Kreth et al., 2008; Koo et al., 2010], while *Actinomyces* spp. preferentially utilize glucose for their metabolism via the Embden-Meyerhof-Parnas pathway [Takahashi, and Yamada, 1999]. Proportions of mFUM, saliva and FBS in the biofilm culture medium were adjusted to 50%, 40% and 10%, respectively, for increasing biofilm density and sturdiness [Ammann et al., 2012]. In contrast to the Zurich biofilm model, biofilms were cultured in 96-well plates rather than on hydroxyapatite discs placed in 24-well plates for ensuring optimal experimental conditions for antimicrobial evaluation of light-based approaches like aPDT. Otherwise, biofilms growing on both sides of the hydroxyapatite discs may have impeded standardized irradiation procedures. Biofilm culture was started with *A. naeslundii* and *A. odontolyticus*, while *S. mutans* was added after 24 h of culture for preventing outgrowth of this species with respect to the slower cell division of *Actinomyces* spp. relative to streptococci [Dige et al., 2009]. Accordingly, it was recently shown that, when *S. oralis* is absent, *S. mutans* dominates the Zurich biofilm at the expense of *A. oris* [Thurnheer, and Belibasakis, 2018]. Furthermore, *S. mutans* colonizes *in vitro* much less efficiently on streptococcal biofilms than on *A. naeslundii* biofilms [Wang et al., 2011].

As compared to the Zurich biofilm model of supragingival plaque and its various modifications comprising up to 11 bacterial or fungal species [Guggenheim et al., 2001; Ammann et al., 2012; Shapiro et al., 2002; Belibasakis, and Thurnheer, 2014], this biofilm model is less complex in terms of species diversity, but on the other hand allows easy and high-throughput screening while still being robust towards antimicrobial challenge. Treatment with CHX 2.0% for 20 min served as a positive

control leading to total eradication of biofilm bacteria below detection limit. In contrast, treatment with CHX 0.2% resulted in a reduction of ≈4 $\log_{10}$ steps while shorter treatment periods exhibited substantially lower inactivation rates (data not shown). This is in line with the literature, where Wilson reported no antimicrobial effect of CHX 0.2% after treating 72 h old *S. mutans* biofilms for 5 min, but eradication by >6 $\log_{10}$ below detection limit after treatment for 60 min [Wilson, 1996]. Likewise, Voos *et al.* found inactivation of ≈1 $\log_{10}$ after treating biofilms formed *in situ* with CHX 0.2% for 3 min [Voos et al., 2014] while Hoogenkamp *et al.* showed ≈2 $\log_{10}$ inactivation of *S. mutans* biofilms after treatment for 5 min [Hoogenkamp et al., 2009]. In general, there is a strong and crucial correlation between treatment period and antimicrobial efficacy especially when it comes to biofilms [Mah, and O'Toole, 2001; Stewart, and Costerton, 2001]. Diffusion of given antimicrobials into deeper layers of the biofilm may be hampered or slowed by interaction of these positively charged molecules with the negatively charged residues of the biofilm's extracellular polymeric substance (EPS) [Tseng et al., 2013; Thurnheer et al., 2003]. Therefore, shorter treatment periods may just lead to killing of bacteria in the superficial layers of the biofilm as it has been shown in the classic study by Zaura *et al.* [Zaura-Arite et al., 2001].

Treatment with aPDT led to less pronounced inactivation rates as compared to CHX. *S. mutans* was inactivated by 2.8 $\log_{10}$ while *A. naeslundii* and *A. odontolyticus* exhibited killing rates of 1.2 or 1.3 $\log_{10}$ only. It is noteworthy to say that despite identical total treatment period of 20 min (10 min of incubation in the dark followed by 10 min of irradiation), the period of antimicrobial activity of aPDT is limited to the time when the light is switched on. Interestingly, although aPDT is known to be an unselective antimicrobial approach [Wainwright et al., 2017], *S. mutans* exhibited noticeably higher susceptibility towards phenalene-1-one mediated aPDT as compared to both, *A. naeslundii* and *A. odontolyticus*. The influence of subsequent addition of *S. mutans*, potentially leading to only superficial colonization of this species within the biofilm, can be ruled out because in this instance *S. mutans* would have been inactivated more severely than both *Actinomyces* spp. in the CHX groups, too. The higher susceptibility of *S. mutans* towards aPDT was further substantiated by investigating antimicrobial efficacy towards monospecies biofilms of all three species. Likewise, aPDT showed higher inactivation efficacy in *S. mutans* biofilms (3.0 $\log_{10}$) as compared to *A. naeslundii* (2.5 $\log_{10}$) and *A. odontolyticus* biofilms (1.1

279 log_{10}). These results are in contrast to a previous study where aPDT with the same
280 PS as used in the present study resulted in a 6.5 log_{10} inactivation against *A.*
281 *naeslundii* monospecies biofilms [Cieplik et al., 2015]. However, this difference in
282 antimicrobial efficacy may be due to employing a distinct strain (*A. naeslundii* T14V)
283 and due to the longer incubation period (25 min) [Cieplik et al., 2015], again
284 elucidating the crucial impact of treatment period.
285 *Actinomyces* spp. are generally known for their thick and robust cell walls, which are
286 resistant to salivary lysozyme and other peptidoglycan-hydrolyzing enzymes [Delisle
287 et al., 2006] Therefore, the higher tolerance towards aPDT may be due to an
288 insufficient permeability for PS molecules through the cell membrane, as suggested
289 by Dige *et al*. as cause for the irregular distribution of fluorescent signals when
290 applying FISH to *A. naeslundii* [Dige et al., 2009]. Furthermore, *Actinomyces* spp.
291 tend to cluster together [Dige et al., 2009] and may even be encapsulated by
292 streptococci as it has been proposed by Bos *et al.* [Bos et al., 1996].
293 To the best of our knowledge, there are no other reports in the literature on the
294 effects of aPDT on polymicrobial biofilms formed *in vitro* from caries-associated
295 species, such as *S. mutans* and *Actinomyces* spp. Therefore, the higher tolerance of
296 *Actinomyces* spp. towards phenalen-1-one mediated aPDT found here may justify
297 more detailed investigation in future studies, *e.g*. by using flow cytometry for
298 investigation of membrane alterations. This phenomenon merits further investigation
299 as it may be utilized clinically by repeated application of aPDT to induce a shift from
300 highly cariogenic streptococci-dominated to less cariogenic *Actinomyces*-dominated
301 microbiota in caries-active patients, which may be an interesting approach for biofilm-
302 modulation in view of the ecological plaque hypothesis [Marsh et al., 2015].
303 However, before any clinical application of phenalen-1-one mediated aPDT potential
304 harmful effects on mammalian tissues must be ruled out. As a first step, in a recent
305 study aPDT with several phenalen-1-one derivatives was investigated for its
306 antimicrobial efficacy towards planktonic cultures of dermal pathogens and for its
307 eukaryotic toxicity towards keratinocytes as compared to the biocide benzalkonium
308 chloride (BAC) in order to evaluate a potential effective concentration range (*i.e*. ≥5
309 log_{10} steps reduction of CFU while ≥80% survival of keratinocytes) [Muehler et al.,
310 2017]. For the PS used in the present study, no harmful effects were found in the
311 dark. In combination with light there was a broad effective concentration range, while
312 for BAC no effective concentration range could be found at all [Muehler et al., 2017].

Although these results are encouraging, further biocompatibility studies will be necessary to establish the clinical safety of phenalen-1-one mediated aPDT.

Conclusion

In this study, a novel caries biofilm model is described comprising *A. naeslundii*, *A. odontolyticus* and *S. mutans*, which allows easy and high-throughput screening of given compounds or approaches while exhibiting sturdiness towards antimicrobial challenges. While treatment with CHX 0.2% inactivated all species by of ≈4 $\log_{10}$ steps, phenalen-1-one mediated aPDT led to more pronounced inactivation of S. mutans (2.8 $\log_{10}$) as compared to *A. naeslundii* (1.2 $\log_{10}$) and *A. odontolyticus* (1.3 $\log_{10}$) which was similarly found in monospecies biofilms.

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All authors declare that they have no conflict of interest.

Author contributions

FC, TM and WB conceived and designed the experiments. TT and GNB helped setting up the biofilm model. FW and DM performed the experiments. FC, FW, DM, KAH, TM and WB analyzed the data. FC wrote the manuscript with input from all authors. All authors reviewed the manuscript.

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Figure Legends

Figure 1: Antimicrobial efficacy on polyspecies biofilms.

All results are shown as medians and neighboring quartiles depicted on a log_{10}-scaled ordinate.

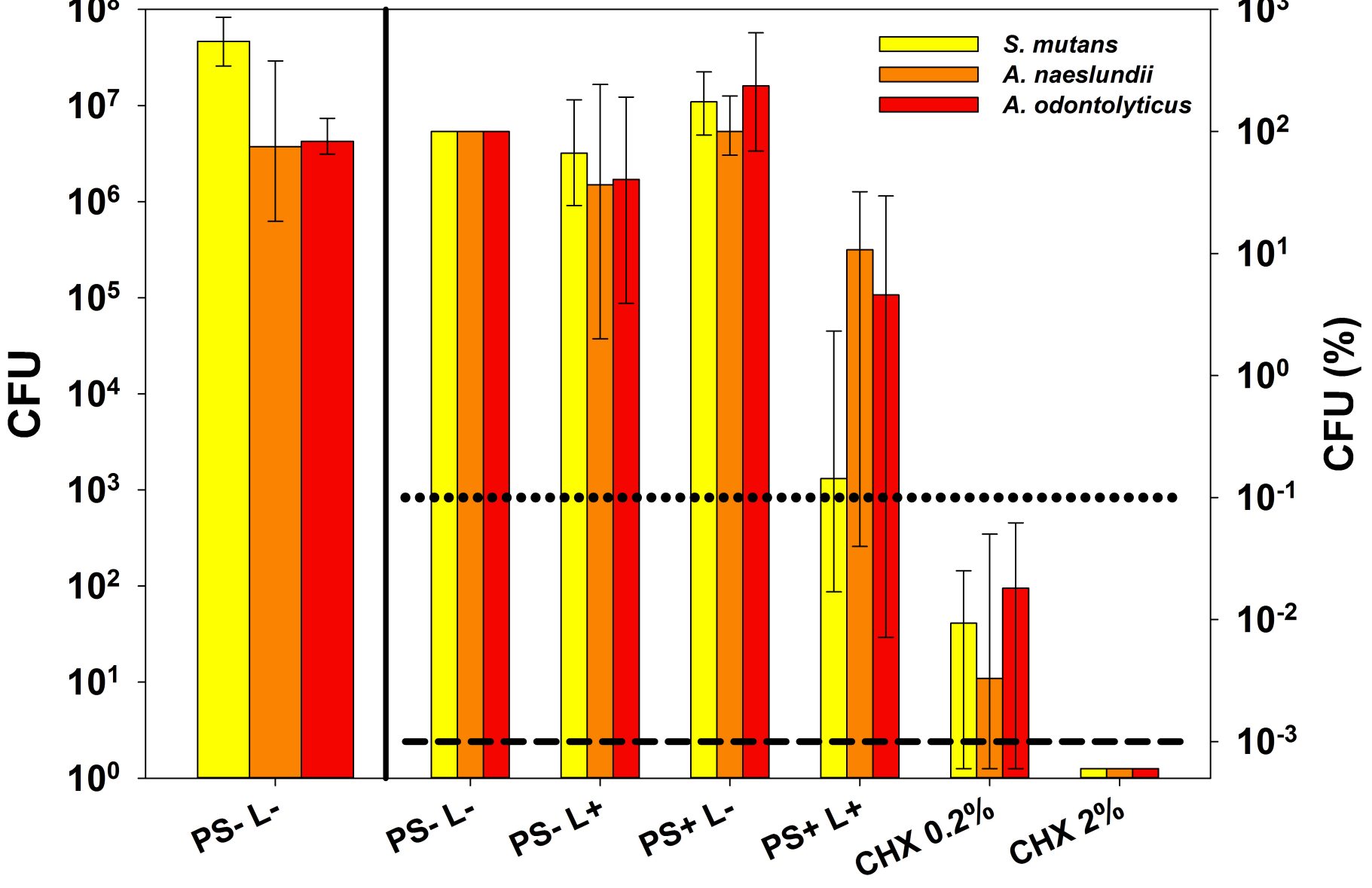
Left panel shows absolute CFU data of untreated control groups (PS-L-) exhibiting over-growth of *S. mutans* of ≈1 log_{10} as compared to both *Actinomyces* spp.

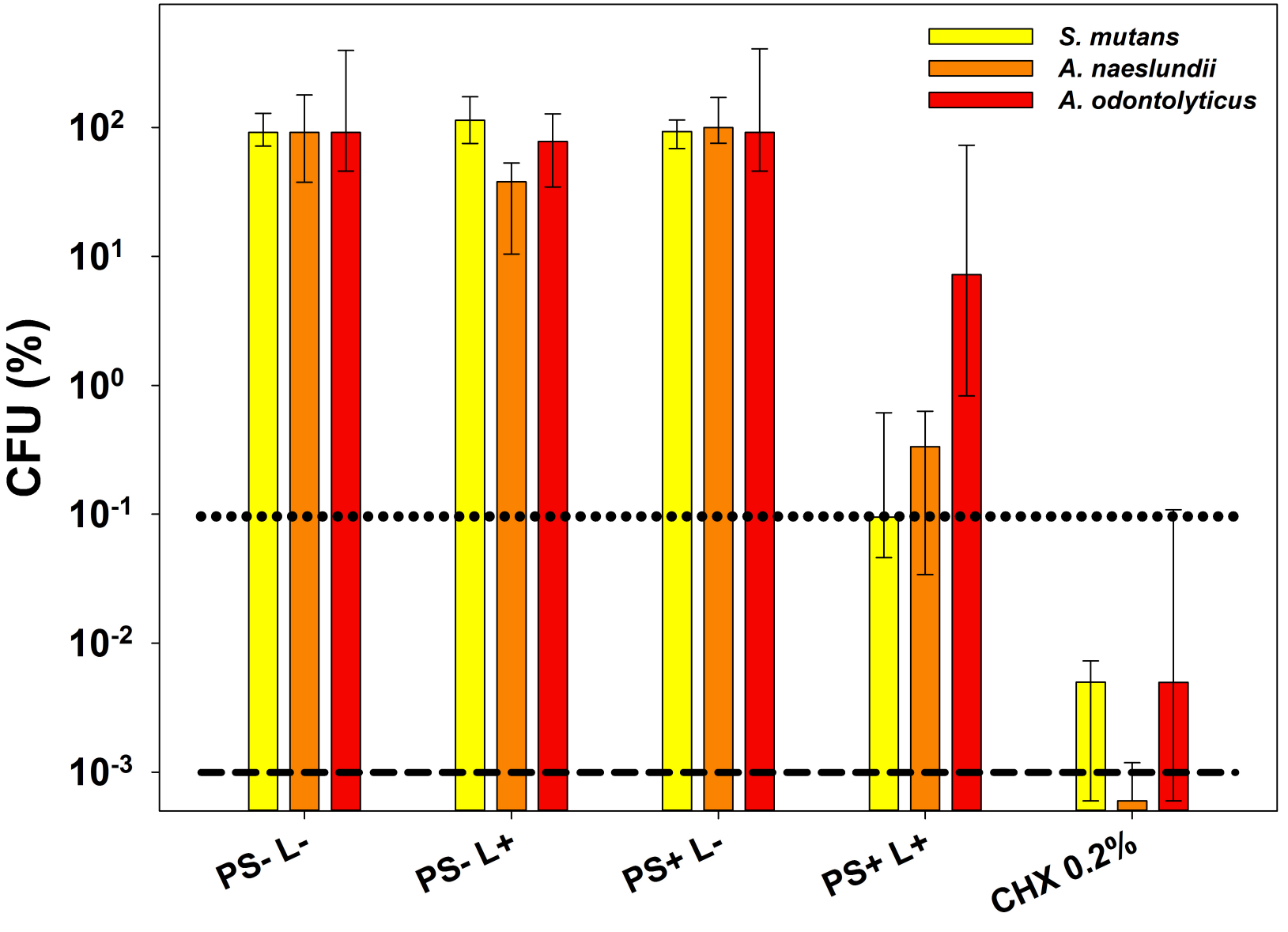
Right panel depicts relative CFU data with untreated controls (groups PS-L-) set to 100%. Horizontal dotted and dashed lines represent CFU-reductions of 3 log_{10} and 5 log_{10}, respectively, as compared to untreated control groups (PS-L-). There was no effect of light (PS-L+) or PS alone (PS+L-). aPDT (PS+L+) led to higher CFU-reduction of *S. mutans* (2.8 log_{10}) than of *A. naeslundii* (1.2 log_{10}) and *A. odontolyticus* (1.3 log_{10}). CHX 0.2% reduced CFU of *S. mutans* by 4.0 log_{10}, *A. naeslundii* by 4.5 log_{10} and *A. odontolyticus* by 3.8 log_{10}. CHX 2% led to reduction of CFU by >6 log_{10} below detection limit.

Figure 2: Antimicrobial efficacy on monospecies biofilms.

All results are shown as medians and neighboring quartiles depicted on a log_{10}-scaled ordinate as relative CFU data with the respective untreated controls (groups PS-L-) set to 100%. Horizontal dotted and dashed lines represent CFU-reductions of 3 log_{10} and 5 log_{10}, respectively, as compared to the respective untreated control groups (PS-L-).

There was no effect of light (PS-L+) or PS alone (PS+L-). aPDT (PS+L+) led to CFU-reductions of 3.0 log_{10} for *S. mutans* biofilms, while CFU in *A. naeslundii* and *A. odontolyticus* biofilms were reduced by 2.5 log_{10} or 1.1 log_{10}, respectively. CHX 0.2% reduced CFU in *S. mutans* and *A. odontolyticus* biofilms by 4.3 log_{10} and in *A. naeslundii* biofilms by 6.0 log_{10}.


CFU
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CFU (%)
10^3
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
S. mutans
A. naeslundii
A. odontolyticus
PS- L-
PS- L-
PS- L+
PS+ L-
PS+ L+
CHX 0.2%
CHX 2%


S. mutans
A. naeslundii
A. odontolyticus
CFU (%)
10²
10¹
10⁰
10⁻¹
10⁻²
10⁻³
PS- L-
PS- L+
PS+ L-
PS+ L+
CHX 0.2%